



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 47/48	A1	(11) International Publication Number: WO 98/56424 (43) International Publication Date: 17 December 1998 (17.12.98)
<p>(21) International Application Number: PCT/GB98/01567</p> <p>(22) International Filing Date: 12 June 1998 (12.06.98)</p> <p>(30) Priority Data: 9712100.8 12 June 1997 (12.06.97) GB 60/054,277 30 July 1997 (30.07.97) US</p> <p>(71) Applicant (for all designated States except US): ML LABORATORIES PLC [GB/GB]; Rutherford Close, Wavertree Technology Park, Liverpool L13 1EJ (GB).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): DUNCAN, Ruth [GB/GB]; Apartment 143B, Chiltem Court, London NW1 1AX (GB). CASSIDY, James [GB/GB]; Larig, Auchmacoy Road, Torphins AB31 4GQ (GB). GERMAN, Lisa [GB/GB]; White Barn, St. Julian's Farm, Old St. Mellons, Cardiff CF3 9YJ (GB). HIRST, Dale [GB/GB]; 85 Daisy Close, Melksham, Wiltshire SN12 6FZ (GB).</p> <p>(74) Agent: HARRISON GODDARD FOOTE; Belmont House, 20 Wood Lane, Leeds LS6 2AE (GB).</p>	<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	
<p>(54) Title: BIOLOGICALLY ACTIVE MATERIALS</p> <p>(57) Abstract</p> <p>A polymer-drug conjugate in which the polymer is the polysaccharide dextrin, linked directly or indirectly to the drug, is effective to deliver the drug to a target site and is biodegradable. The conjugate may be prepared by succinoylating dextrin followed by reaction with the drug or a derivative thereof.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

BIOLOGICALLY ACTIVE MATERIALS

FIELD OF THE INVENTION

5 This invention relates to biologically active materials, and, in particular, to materials which comprise a polymer linked to a biologically active agent. The invention is concerned with materials known as polymer-drug conjugates which typically contain a therapeutic agent, for instance, a bioactive cytotoxic drug, linked to a polymer backbone. The linkage between the polymer and the drug is typically by covalent
10 bonding. However the invention is applicable to other polymer conjugates including those where the biologically active agent is an imaging agent, such as tyrosinamide, a diagnostic agent, or a targeting agent, such as biotin.

Reference will be made hereinbelow to polymer-drug conjugates and, more
15 particularly, to those in which the drugs are anticancer agents. However, the present invention has application in connection with other drugs and/or bioactive agents.

BACKGROUND OF THE INVENTION

20 In designing a polymer-drug conjugate, the aim is to deliver a drug effectively to a therapeutic site such as a tumour. It is known, for instance, that polymer-drugs given intravenously can accumulate selectively in solid tumour tissue by the EPR effect.

The most commonly used anticancer agents are low molecular weight compounds
25 which readily gain access to cells by rapid passage across the cell membrane. After intravenous (IV) administration, a large percentage of the injected dose leaves the circulation within a few minutes, resulting in a ubiquitous body distribution of drug and little selective concentration in tumour tissue. By creating a macromolecular polymer-anticancer drug conjugate, there is provided an opportunity to improve
30 tumour specific targeting, to minimise drug entry into sites of toxicity, to control

precisely the rate of drug liberation at the target site (giving opportunities for long-term controlled release) and deliver the active principal intracellularly, providing a means to overcome p-glycoprotein related multidrug resistance.

- 5 Numerous polymers have been proposed for synthesis of polymer-drug conjugates including polyaminoacids, polysaccharides such as dextran, and synthetic polymers such as N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer. However, these polymers have limitations. For example, a dextran-doxorubicin conjugate has been tested clinically and been found to be much more toxic than the parent drug and the
- 10 HPMA copolymers which have been clinically tested have the disadvantage of being non-biodegradable in the main chain.

STATEMENTS OF INVENTION

- 15 The present invention provides a polymer-drug conjugate in which the polymer is the polysaccharide dextrin. The polymer-drug conjugate may be one in which the polymer is linked directly to the drug or one in which the polymer is linked indirectly to the drug, for instance, by means of a "biodegradable spacer" to which both the drug and the polymer are linked. The dextrin is preferably a non-cyclic dextrin.

20

- It has been found that dextrin is not only a suitable material for forming a biocompatible polymer-drug conjugate capable of delivering a drug to a target site and of releasing the drug at such a site but is also biodegradable in a manner such that it may be used at a molecular weight which is suitable for the particular drug and
- 25 its application without any upper limit imposed by the need to ensure excretion of the polymer.

- The term "dextrin" means a glucose polymer which is produced by the hydrolysis of starch and which consists of glucose units linked together by means mainly of alpha-
- 30 1,4 linkages. Typically dextrans are produced by the hydrolysis of starch obtained from various natural products such as wheat, rice, maize and tapioca. In addition to

alpha-1,4 linkages, there may be a proportion of alpha-1,6 linkages in a particular dextrin, the amount depending on the starch starting material. Since the rate of biodegradability of alpha-1,6 linkages is typically less than that for alpha-1,4 linkages, for many applications it is preferred that the percentage of alpha-1,6 linkages is less than 10% and more preferably less than 5%.

Any dextrin is a mixture of polyglucose molecules of different chain lengths. As a result, no single number can adequately characterise the molecular weight of such a polymer. Accordingly various averages are used, the most common being the weight average molecular weight (Mw) and the number average molecular weight (Mn). Mw is particularly sensitive to changes in the high molecular weight content of a polymer whilst Mn is largely influenced by changes in the low molecular weight of the polymer.

It is preferred that the Mw of the dextrin is in the range from 1,000 to 200,000, more preferably from 2,000 to 55,000.

The term 'degree of polymerisation' (DP) can also be used in connection with polymer mixtures. For a single polymer molecule, DP means the number of polymer units. For a mixture of molecules of different DP's, weight average DP and number average DP correspond to Mw and Mn. In addition DP can also be used to characterise a polymer by referring to the polymer mixture having a certain percentage of polymers of DP greater than a particular number or less than a particular number.

It is preferred that, in the dextrin-drug conjugate of the present invention, the dextrin contains more than 15 % of polymers of DP greater than 12 and, more preferably, more than 50% of polymers of DP greater than 12.

The drug loading on the polymer is preferably from 0.5 to 99.5 mole %.

A targeting group may be attached either directly or indirectly to the polymer of the conjugate. It is preferred that the ratio of drug to targeting group is from 1:99 to 99:1.

- 5 Preferably the dextrin used in a dextrin-drug conjugate of the present invention is water soluble or at least forms a suspension in water.

The dextrin of a dextrin-drug conjugate of the invention may be in the form of either unsubstituted dextrin (as obtained by the hydrolysis of the starch) or may be substituted by one or more different groups. The substituents may be negatively charged groups, for instance, sulphate groups, neutral groups or positively charged groups, for instance, quaternary ammonium groups. In the case where the substituent group is sulphate, it is preferred that the sulphated polysaccharide contains at least one sulphate group per saccharide (glucose) unit. A particular dextrin sulphate is
15 dextrin-2-sulphate.

Examples of drugs which may form suitable conjugates with dextrin are:- alkylating agents such as cyclophosphamide, melphalan and carmustine; antimetabolites such as methotrexate, 5-fluorouracil, cytarabine and mercaptopurine; natural products such as
20 anthracyclines (eg daunorubicin, doxorubicin and epirubicin), vinca alkaloids (eg vinblastine and vincristine) as well as dactinomycin, mitomycin C, taxol, L-asparaginase and G-CSF; and platinum analogues such as cisplatin and carboplatin.

The present invention also provides a pharmaceutical composition comprising a
25 dextrin-drug conjugate and a pharmaceutically acceptable excipient or diluent therefor.

In addition, the present invention provides the use of a polymer-drug conjugate of the invention in the treatment of a medical condition in connection with which the drug
30 is effective. Furthermore, the invention provides the use of a polymer-drug

conjugate of the invention in the manufacture of a medicament for use in the treatment of a medical condition in connection with which the drug is effective.

- 5 The present invention also provides a method of treating an animal subject, including a human being, including treating the animal subject with a pharmaceutically effective dose of a dextrin-drug conjugate. The conjugate may be administered by any appropriate method, for instance, intravenously, intraperitoneally, orally, parentally or by topical application.
- 10 Various methods have been proposed for the preparation of polymer-drug conjugates. Within the scope of the present invention is a method comprising succinoylating dextrin and reacting the succinoylated dextrin with a drug or reactive derivative thereof.
- 15 Preferably, the dextrin is dissolved in anhydrous dimethyl formamide and is contacted with dimethyl amino pyridine and succinic anhydride. The resultant mixture is then purged with an inert gas and chemical reaction is allowed to take place over a prolonged period, preferably at least 12 hours.
- 20 The resultant succinoylated dextrin is reacted with a drug or drug derivative eg. doxorubicin hydrochloride to form the polymer-drug conjugate.

BRIEF DESCRIPTION OF THE DRAWINGS

- 25 **FIG. 1** is a graphical illustration of the effect of dextrans on red blood cell lysis. **FIG. 2** is a graphical illustration of the effect of compounds MLD 15/73, MLD 5/29 and DXO 40G against Poly L lysine and Dextran.
- FIGS. 3A and 3B** illustrate the synthetic route for the preparation of dextrin-doxorubicin.
- 30 **FIG. 4** is a graphical illustration of the cytotoxicity of doxorubicin and a dextrin-dox conjugate against L1210 cells (72h).

FIG. 5 is a graphical illustration of tumour levels of doxorubicin after administration of dextrin-doxorubicin and free doxorubicin.

FIG. 6 illustrates an electrophoresis curve for the crude and purified radiolabelled dextrin-tyrosinamide.

- 5 FIGS. 7A, 7B and 7C illustrate body distribution of ¹²⁵I-labelled dextrin (MW 51000) at 5 minutes, 30 minutes and 1 hour respectively.

FIG. 8 illustrates the elution of radioactivity from a PD10 Column (Sephadex G-25).

FIG. 9 illustrates the stability of dextrans of different molecular weight in Saline Phosphate Buffer at pH 7.4 without enzymes.

- 10 FIG. 10 illustrates the stability of dextrans of different molecular weight in Salt Citrate Buffer at pH 5.5 without enzymes.

FIG. 11 illustrates DX0 40G degradation and low Mw saccharides release profiles during incubation in rat plasma at 37 C.

- 15 FIG. 12 illustrates MLD 15/73 degradation and low Mw saccharides release profiles in rat plasma at 37 C.

FIG. 13 illustrates degradation of dextrin MD15/73.

EXAMPLES OF THE INVENTION

- 20 The present invention will now be further described with reference to the following examples. In these examples three dextrin materials are used, their molecular weight characteristics being as follows (Table 1):-

25

Code	Mw	Mn	Mw/Mn
MLD5/29	5K	6K	2.74
DX04/0G	15K	6K	2.74
MLD15/73	51K	28K	1.84

Table 1: Examples confirming the Biocompatibility of Dextrans

Example 1: Effect of Dextrins on the stability of rat red blood cells incubated in vitro.

Method:- Dextrins of different molecular weight were incubated with isolated rat red blood cells (RBC) and haemoglobin release detected spectrophotometrically. Various dilutions of the test polymers (dextrins) and of the reference polymer polyethyleneimine were made up in PBS and mixed with an equal volume of fresh 2% w/v suspension of rat RBCs. This preparation was centrifuged at 1500 x g for 10 min at room temperature. Then, 100 µl of the supernatant was removed and the levels of haemoglobin quantitated spectrophotometrically at 550 nm. The results illustrated in Figure 1 were expressed as % lysis relative to that of a Triton X-100 control (Sigma) which gave 100% RBC lysis.

Results:- None of the dextrin polymers induced red cell lysis. Polyethyleneimine and the detergent Triton X-100 (control 100% lysis) both caused haemoglobin release.

Example 2: Cytotoxicity of unmodified dextrins against L1210 cells.

Method:- L1210 cells were seeded at a density of 1×10^4 cells per well into 96 well 'v' bottomed microtiter plates (Costar) in RPMI 1640 tissue culture media (Gibco) supplemented with 10% Foetal Calf Serum (FCS) (Gibco). All incubations were carried out at 37°C in an atmosphere of 5% CO₂.

The cells were then left for 24h. Prior to being incubated with the polymers, the cells were centrifuged at 1000 x g for 10 minutes at room temperature. This served to pellet the cells and allow the removal of the media. The polymers were then dissolved in RPMI 1640 containing 10% FCS and filter sterilised through 0.2 µ filters (Aerodisk). Following this, the pellet was resuspended in media containing the appropriate concentration of the dextrins of different molecular weight. Positive and negative reference controls were incorporated, dextran Mw 72 000 (Sigma) as a negative control and poly(L-Lysine) Mw 56 500 (Sigma) as a positive control. These

polymers were added to cells at the same concentrations as the test polymers. The tetrazolium dye MTT was used to assess cell viability. The cells were then incubated for 67h prior to the addition of the MTT. After a further incubation period of 5h, the cells in suspension were subjected to centrifugation, the media removed and 100 μ l of optical grade DMSO (Sigma) added. The plates were read at 550 nm using a Titerteck plate reader. The results are illustrated in Figure 2. The % viabilities are optical densities (OD's) expressed as a % of the OD seen in cell cultures containing no polymer.

- 10 **Results:-** None of the dextrans was cytotoxic. The toxic effect of the reference control poly-L-Lysine can be seen.

Examples Relating to Dextrin Modification for coupling of pendent groups

- 15 To bind pendent drugs or side chains to dextrans there are a number of possible activation methods that may be used. These include:

a) **Periodate oxidation:** This is a ring opening method resulting in polyaldehyde derivatives of a polysaccharide (Bruneel and Schacht 1992).
20 Unfortunately this reaction is prone to a number of side reactions that makes the production of single, pure, easily characterised compound quite difficult. In particular there is evidence for the formation of intra-residual hemiacetal formation. However, when formed aldehyde groups represent sites where amines can be attached to the polymer in the form of Schiff base structures or alkyl amines.

25

b) **The 4-nitrophenyl chloroformate method:** This is easily carried out at 0°C and there is much information available on the analysis of the various groups that do, or do not, react. Clean up/purification procedures would appear to be straightforward and percentage reactivity of active groups in the range of interest would appear to be
30 controllable to an effective extent. There is some concern, however, over the

formation of undesirable five membered cyclic carbonates (Bruneel and Schacht 1992).

5 c) **Succinylation:** Activation by succinoylations is also an easily performed operation. The conditions employed are however somewhat acerbic by comparison with the chloroformate method. The initial reaction requires refluxing at 40°C for 24hr in dry DMSO, but results in the formation of carbonate functionalities. This reaction also includes an extra step on the way to the production of the amine derivative by virtue of going through an intermediate step involving N,N'-
10 carbonyldiimidazol. Here too, purification would appear to be relatively uncomplicated.

d) **Cyanogen Bromide activation:** Activation by cyanogen bromide is easily carried out in aqueous conditions at room temperatures. (Immobilised Affinity
15 Ligand Techniques, Herman GT, Mallia AK and Smith PK. Pub: Academic Press, New York, 1992). Excess reagent must however be neutralised and eliminated as it may liberate hydrogen cyanide in an acidic environment. In addition to this side reactions may occur resulting in several reactive intermediates. It does however have advantages in that a primary amine can be introduced directly on the reactive
20 intermediate. Finally there is evidence for the linkage being unstable resulting in slow release of the pendant group which may or may not be advantageous or desirable.

Examples: Modification of dextrans by succinylation and reaction with
25 **compounds containing amino groups (in relation to (c) above).**

All three dextrin samples were modified by means of succinylation. Initially, attempts to modify the samples to the extent of 50, 20, 10, 5, 2.5 and 1 mol% were carried out. The sample with the highest molecular weight was taken forward for further reaction with tyrosinamide and doxorubicin.

Example 3.1: Synthesis of Succinoylated Dextrins.

In triplicate batches of dextrin (1g, MD 15/73, 1.94×10^{-3} mol) was dissolved in anhydrous dimethyl formamide (DMF, 10 ml). Dimethyl amino pyridine (DMAP, 28.3 mg) was then added followed by succinic anhydride (62 mg, 6.2×10^{-4} mol, 10 mol %). The mixture was then purged with nitrogen, sealed and left to stir for 24 h at temperatures of 20, 30, 40 and 50°C. Following this the reaction mixture was poured onto rapidly stirring diethyl ether (100 ml) and magnetically stirred overnight. The ether was then removed by filtration under vacuum and the resulting solid dissolved into a minimum of distilled water poured into a dialysis membrane (Visking tubing, MW cut-off 12000-1400) and purified by dialysis against 2 x 2L of distilled water. The resulting solution was freeze dried and recovery calculated. Degree of succinoylation was determined by titration against a relevant standard of NaOH. After application of the students T-Test there was no difference in the degree of succinoylation for any reaction condition. Results are shown in Table 2.

Table 2. Results of the effect of temperature on the succinoylation of dextrin

Temperature	% Recovery (g) (\pm SD)	Mol % Succinoylation (\pm SD)
20 *	ND	ND
30	53.3 (6.7)	6.64 (0.74)
40	73.4 (10.7)	5.49 (0.32)
50	42.6 (12.3)	2.26 (0.12)

* Did not dissolve

As an alternative procedure the product may be purified by gel filtration, ultrafiltration or centrifugation against a pore controlled membrane.

The same reaction conditions (adjusting the ratios of the components or solvents) were used to achieve degrees of succinoylation of the dextrin samples of different molecular weights (MW 4950, 15000 and 51,100). See Table 1.

Example 3.2: Conjugation of Doxorubicin to Succinoylated Dextrins.

Succinoylated dextrin (51 mg, 1×10^{-6} mol, MD 15/73, 5.3 mol % succinoylation) was dissolved in 2 ml of distilled water, to this was added EDC (3.51 mg 1.8×10^{-5} mol). This was allowed to react at room temperature while gently stirring for 30 min.

5 After this time Doxorubicin hydrochloride (Pharmacia) was added (9.67 ml of a 1 mg ml^{-1} solution, 1.67×10^{-5} mol). The reaction vessel was wrapped in foil and allowed to stir overnight at room temperature. The product was isolated by solvent extraction with chloroform and passage over a LH 20 superdex column (Pharmacia) followed by freeze drying to constant weight. Finally free and conjugated Doxorubicin was

10 measured by extractive HPLC. Results of various conjugations are summarised in Table 3.

Table 3. Incorporation of Doxorubicin on various Dex-Dox Conjugates

Succinoylation Mol %	Coupling Conditions	Time	Percent W/W incorporation
0.5	EDC/Water	Overnight	0.127
6.53	EDC/Water	Overnight	4.2
6.53	CDI/DMF	Overnight	3.9
5.3	CDI/DMF	4 hours	2.1
14.9	EDC/water/Sulpho-NHS	4 hours	6.39
14.9	EDC/WATER	4 hours	2.82

15 Alternatively Doxorubicin may be conjugated using Sulpho-NHS and EDC in aqueous conditions to improve coupling yields or with other peptide coupling agents and solvent of choice.

The synthetic route for the preparation of dextrin-doxorubicin is illustrated in Figures

20 3a and 3b.

Example 3.3: Conjugation of Tyrosinamide to Succinoylated Dextrin.

Succinoylated dextrin (57.7mg, MD 15/73, 1 mol%) was dissolved in 3ml of DMF, to this was added CDI (10.4mg, 6.4×10^{-5} mol) dissolved in DMF (1ml). This was

25 allowed to react at 25°C while stirring for 1 h. After this time tyrosinamide (97mg,

8.96 x 10⁻⁴ mol was dissolved in DMF (1ml) and added to the reaction. This was allowed to react stirring at room temperature at 25°C for 48 h. The product was isolated by removal of the DMF by rotary evaporation. To the product was added water (3ml) followed by freeze drying until constant weight was maintained. The product was purified by dialysis against distilled water (24 h).

Example 3.4: Conjugation of Biotin hydrazide to Succinoylated Dextrin.

Succinoylated dextrin (57.7mg, MD 15/73, 1 mol %) was dissolved in 3ml of DMF, to this was added CDI (10.4mg, 6.4 x 10⁻⁵ mol) dissolved in DMF (1ml). This was allowed to react at 25°C while stirring for 1 hour. After this time biotin hydrazide (97mg, 8.96 x 10⁻⁴ mol) was dissolved in DMF (1ml) and added to the reaction. This was allowed to react stirring at room temperature at 25°C for 48 hour. The product was isolated by removal of the DMF using by rotary evaporation. To the product was added water (3ml) followed by freeze drying until constant weight was maintained. The product was purified by dialysis against distilled water (24 h).

Example 4: Evaluation of the cytotoxicity of doxorubicin and dextrin-doxorubicin conjugates.

Method:- L1210 cells were seeded at a density of 1x10⁴ cells per well into 96 well 'v' bottomed microtiter plates (Costar) in RPMI 1640 tissue culture media (Gibco) supplemented with 10% Foetal Calf Scrum (FCS) (Gibco). All incubations were carried out at 37°C in an atmosphere of 5% CO₂.

The cells were then left for 24h. Prior to being incubated with the polymers the cells were centrifuged at 1000 x g for 10 minutes at room temperature. This served to pellet the cells and allow the removal of the media.

Doxorubicin and dextrin doxorubicin were then dissolved in RPMI 1640 containing 10% FCS and filter sterilised through 0.2µ filters (Aerodisk). Following this, the pellet was resuspended in media containing the appropriate concentration of drug or drug conjugate.

The tetrazolium dye MTT was used to assess cell viability. The cells were incubated for 67h prior to the addition of the MTT. After a further incubation period of 5h, the cells in suspension were subjected to centrifugation, the media removed and 100 µl of optical grade DMSO (Sigma) was added. The plates were read at 550 nm using a
5 Titerteck plate reader. Results (OD) were expressed as a % of the OD seen in cell cultures containing no polymer. They are illustrated in Figure 4.

Results:- It can be seen that both doxorubicin and the dextrin doxorubicin conjugate (prepared in example 3.2) were toxic to L1210 cells in vitro. This confirms that
10 active doxorubicin can be released from the conjugate over the 72h incubation period. It is not surprising that the conjugate is less active than the parent drug in vitro. This is a well known phenomenon attributed to the very slow capture of a polymeric drug by cells by the mechanism of endocytosis. Free doxorubicin penetrates the cell immediately by passing across the cell membrane.

15

Example 5: Experiments to demonstrate the targeting on dextrin-doxorubicin to sc. B16F10 (mouse melanoma).

Method:- C57 black mice were injected subcutaneously (sc) with 10^5 B16F10 melanoma cells in normal saline (100 µl). The mice were checked daily for the
20 appearance of tumours and when palpable (10-13 days) the mice were then injected iv with dextrin-doxorubicin (6.0 wt % dox) at a dose of 5 mg/kg dox-equivalent. The mice were culled after 1 min, 1 and 24 h and the tumours were removed, weighed and homogenised in double distilled water (2 ml). The total doxorubicin content was measured by HPLC after acid hydrolysis and extraction into 4:1 chloroform :
25 isopropyl alcohol. Doxorubicin content was then expressed as µg dox/per g of tumour.

Results:- After administration of dextrin-doxorubicin no signs of toxicity were observed. It can be seen that administration of drug as a dextrin conjugate resulted in
30 significantly higher tumour levels than seen following administration of free drug (Figure 5).

Example 6: Radiolabelling of Dextrin-tyrosinamide.

Method:- To allow monitoring of body distribution and rate of degradation the highest molecular weight dextrin-TyrNH₂ was labelled using the Chloramine T
5 method.

Dextrin-tyrosinamide (5mg) was dissolved in phosphate buffer (0.5ml, 0.05M, pH7.0. Na[¹²⁵I]iodide (5µl, 5mCi) was added to the reaction vessel and was left stirring for 2min. Chloramine T (75µl, 2mg/ml) was added and left stirring for
10 15min. Sodium metabisulphate (500µl, 2mg/ml) and a crystal of potassium iodide was added to the reaction vessel and left for 2 min.

Specific activity and purity was determined by paper electrophoresis. Whatman chromatography paper (5 x 30 cm strips) were divided into 5mm strips by pencil (40
15 strips). Marking the fifth strip as the point of application for the sample. The paper was soaked in barbitone buffer (Sigma B6632) and then blotted dry. Into a paper electrophoresis tank (Shandon) was placed the same buffer and the paper was placed on the supporting bars. Na[¹²⁵I]iodide (4µl) was placed on the application point nearest the anode as a reference and the crude and pure radiolabelled polymer (4µl)
20 was placed on the application point nearest the anode as a reference and the crude and pure radiolabelled polymer (4µl) was loaded onto separate strips. The tank was connected to a power supply and the samples run for 30min at 400V (constant voltage). The chromatography papers were removed and each 5mm strip was cut, and placed into counting tubes with 1ml of water in each and the presence of
25 radioactivity was measured using a gamma counter (Packard). The results were plotted as counts per minute against distance migrated. Results are illustrated in Figure 6.

Results:- The dextrin product was radiolabelled and had a specific activity of
30 approximately 20µCi/mg.

Example 7: Body distribution of ^{125}I -labelled dextrin.

Adult male Wistar rats were anaesthetised with a mixture of oxygen and isoflurothane and radiolabelled dextrin-tyrosinamide (100 μl , specific activity 11.8 $\mu\text{Ci/mg}$) were injected iv, ip or subcutaneous). The rats were culled after the
5 desired time using carbon dioxide. The rat was weighed and all major organs (lungs, heart, liver, kidneys, spleen, thyroid, urine, bladder or blood) were removed. After ip administration a peritoneal wash was also taken. The organs were homogenised in water and samples (1ml in triplicates) were counted in the gamma counter. The results expressed as % dose injected for each organ are illustrated in Figures 7a, 7b
10 and 7c.

Samples of the urine were subjected to gel permeation chromatography (GPC) using a PD10 column to evaluate the degradation of the polymer in the body. Results are shown in Figure 8.

15

Results:- After 1h most of the radioactivity recovered following both ip and iv injection was detected in the urine and faeces. GPC analysis of urine samples indicated liberation of low molecular weight degradation products.

20 **Example 8: Evaluation of the rates of hydrolytic and enzymatic degradation of dextrin samples of different molecular weight.**

Experiments were carried out to determine the stability of the three dextrin samples in physiological buffers (pH 7.4 and 5.5) and also in the presence of enzymes (plasma and lysosomal enzymes (tritosomes).

25

The molecular weight of the samples and changes with time were monitored by gel permeation chromatography. Two types of experiment were undertaken.

30 **Example 8.1:- Degradation of native and modified non-radiolabelled dextrans by enzymes followed by HPLC/GPC with RI detection.**

Method:- GPC size exclusion was carried out using a TSK G5000 PW and G2000 PW columns in series (7.5 x 300 mm). The sample injection loop was 20 μl , the

mobile phases used were saline phosphate buffer (PBS) at pH 7.4 and later high salt (NaCl 0.25M) citrate buffer at pH 5.5 made with double distilled water in both cases. The eluant flow rate in all cases was 1ml min⁻¹.

- 5 Column calibration was carried out with pullulan and glucose molecular weight standards. The method of detection was by means of differential refractometry using a Knauer refractive index detector.

10 Samples (at concentration of approximately 5mg ml⁻¹) were incubated in the appropriate buffer (with or without enzymes) for several days at 37°C. At various time periods a small sample was taken and subjected to GPC.

Results:- In the absence of enzyme, the dextrin samples at 37°C did not show any evidence of hydrolysis. Figures 9 and 10 illustrate the stability of dextrin of different molecular weights in buffers without enzymes. When plasma enzymes were added samples degraded rapidly over the first hour and more slowly thereafter. Addition of lysosomal enzymes did not cause dextrin degradation. Figures 11 and 12 illustrate for samples DX0409 and MLD15/73 respectively, the dextrin degradation and low molecular weight saccharides release during incubation in rat plasma at 37°C.

20

Example 8.2: Degradation of ¹²⁵I-labelled dextrin measured by PD10 GPC.

Method:- The radiolabelled dextrin sample prepared for the body distribution studies was also incubated with plasma and lysosomal enzymes and samples subjected to PD10 Sephadex chromatography to evaluate the rate of degradation. Fractions eluting from the column were assayed for radioactivity and the results expressed as the percentage decrease in peak one (the polymer peak). The results for dextrin sample MD15/73 are illustrated in Figure 13.

Results:- Although the polymer sample degraded rapidly in the presence of plasma enzymes the product appeared stable in the presence of lysosomal enzymes.

15182.3

CLAIMS

1. A polymer-drug conjugate in which the polymer is the polysaccharide dextrin which is linked either directly or indirectly to the drug.
- 5 2. A polymer-drug conjugate according to claim 1 in which the drug is doxorubicin.
3. A polymer-drug conjugate according to claim 1 or claim 2 in which the dextrin is a non-cyclic dextrin.
- 10 4. A polymer-drug conjugate according to any of the preceding claims in which the percentage of alpha-1,6 linkages in the dextrin is less than 10%.
- 15 5. A polymer-drug conjugate according to claim 4 in which the percentage of alpha-1,6 linkages in the dextrin is less than 5%.
6. A polymer-drug conjugate according to any of the preceding claims in which the weight average molecular weight of the dextrin is in the range from 1,000 to 200,000.
- 20 7. A polymer-drug conjugate according to claim 6 in which the weight average molecular weight of the dextrin is in the range from 2,000 to 55,000.
- 25 8. A polymer-drug conjugate according to any of the preceding claims in which the dextrin contains more than 15% of polymers of DP greater than 12.
9. A polymer-drug conjugate according to claim 8 in which the dextrin contains more than 50% of polymers of DP greater than 12.
- 30 10. A polymer-drug conjugate according to any of the preceding claims in which the drug loading on the polymer is from 0.5 to 99.5 mole %.

11. A polymer-drug conjugate according to any of the preceding claims in which a targeting group is attached either directly or indirectly to said polymer.
- 5 12. A polymer-drug conjugate according to claim 11 in which the ratio of drug to targeting group is from 1 : 99 to 99 : 1.
13. A polymer-drug conjugate according to any of the preceding claims in which the dextrin is water soluble or at least forms a suspension in water.
- 10 14. A polymer-drug conjugate according to any of the preceding claims in which the dextrin used is in the form of unsubstituted dextrin.
- 15 15. A polymer-drug conjugate according to any of the preceding claims in which the dextrin is substituted by negatively charged, neutral, or positively charged substituent groups.
16. A polymer-drug conjugate according to claim 15 wherein the substituent group is sulphate.
- 20 17. A polymer-drug conjugate according to claim 16 wherein the sulphated polysaccharide contains at least one sulphate group per saccharide (glucose) unit.
- 25 18. A pharmaceutical composition comprising the dextrin-drug conjugate of any of the preceding claims and a pharmaceutically acceptable excipient or diluent therefor.
19. A pharmaceutical composition according to claim 18 in the form of an aqueous solution or suspension.

30

20. Use of the polymer-drug conjugate of any of claims 1 to 19 in the treatment of a medical condition in connection with which the drug is effective.
- 5 21. Use of a polymer-drug conjugate of any of claims 1 to 19 in the manufacture of a medicament for use in the treatment of a medical condition in connection with which the drug is effective.
- 10 22. A method of treating an animal subject, including a human being, the method comprising treating the animal subject with a pharmaceutically effective dose of the dextrin-drug conjugate of any of claims 1 to 17.
23. A method according to claim 22 in which the conjugate is administered intravenously, intraperitoneally, orally, parenterally or by topical application.
- 15 24. A method of preparing a polymer-drug conjugate as claimed in any of claims 1 to 23, the method comprising succinoylating dextrin and reacting the succinoylated dextrin with a drug or reactive derivative thereof.
- 20 25. A method according to claim 24 wherein the dextrin is dissolved in anhydrous dimethyl formamide.
26. A method according to claim 25 wherein the dissolved dextrin is contacted with dimethyl amino pyridine and succinic anhydride.
- 25 27. A method according to claim 26 wherein the resultant mixture is purged with an inert gas.
28. A method according to claim 27 wherein chemical reaction is allowed to take place over a prolonged period.
- 30 29. A method according to claim 28 wherein said period is at least 12 hours.

30. A method according to any of claims 24 to 29 wherein the succinoylated dextrin is reacted with doxorubicin hydrochloride to form the polymer-drug conjugate.

5

1/15

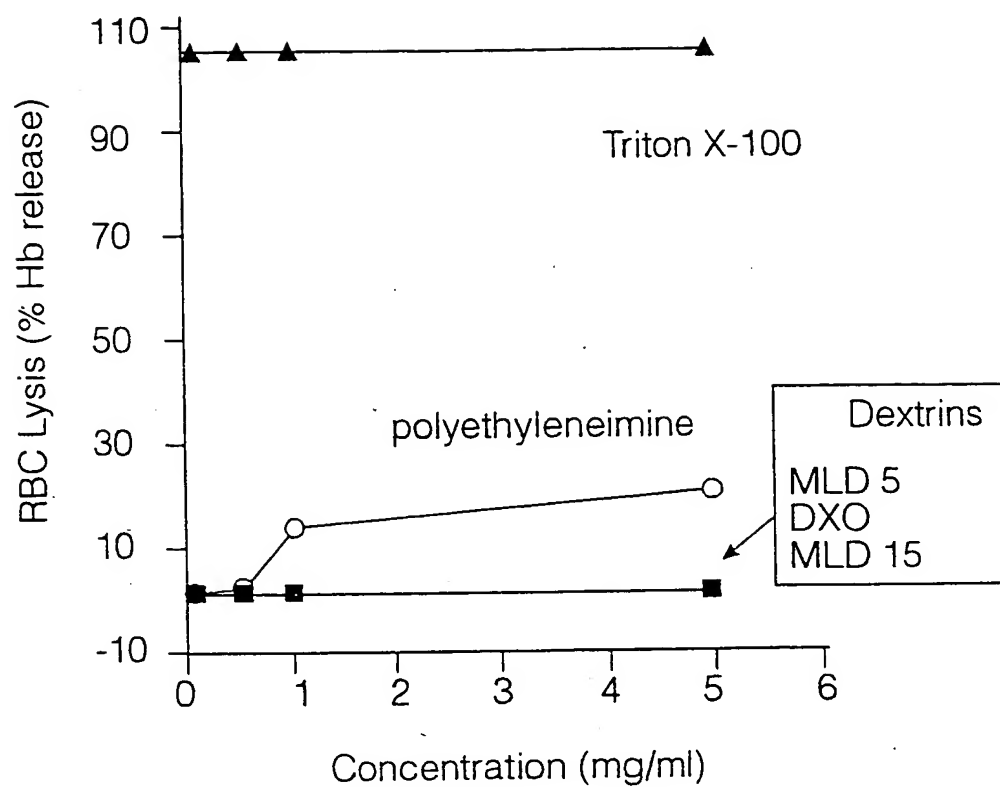


Fig. 1

2/15

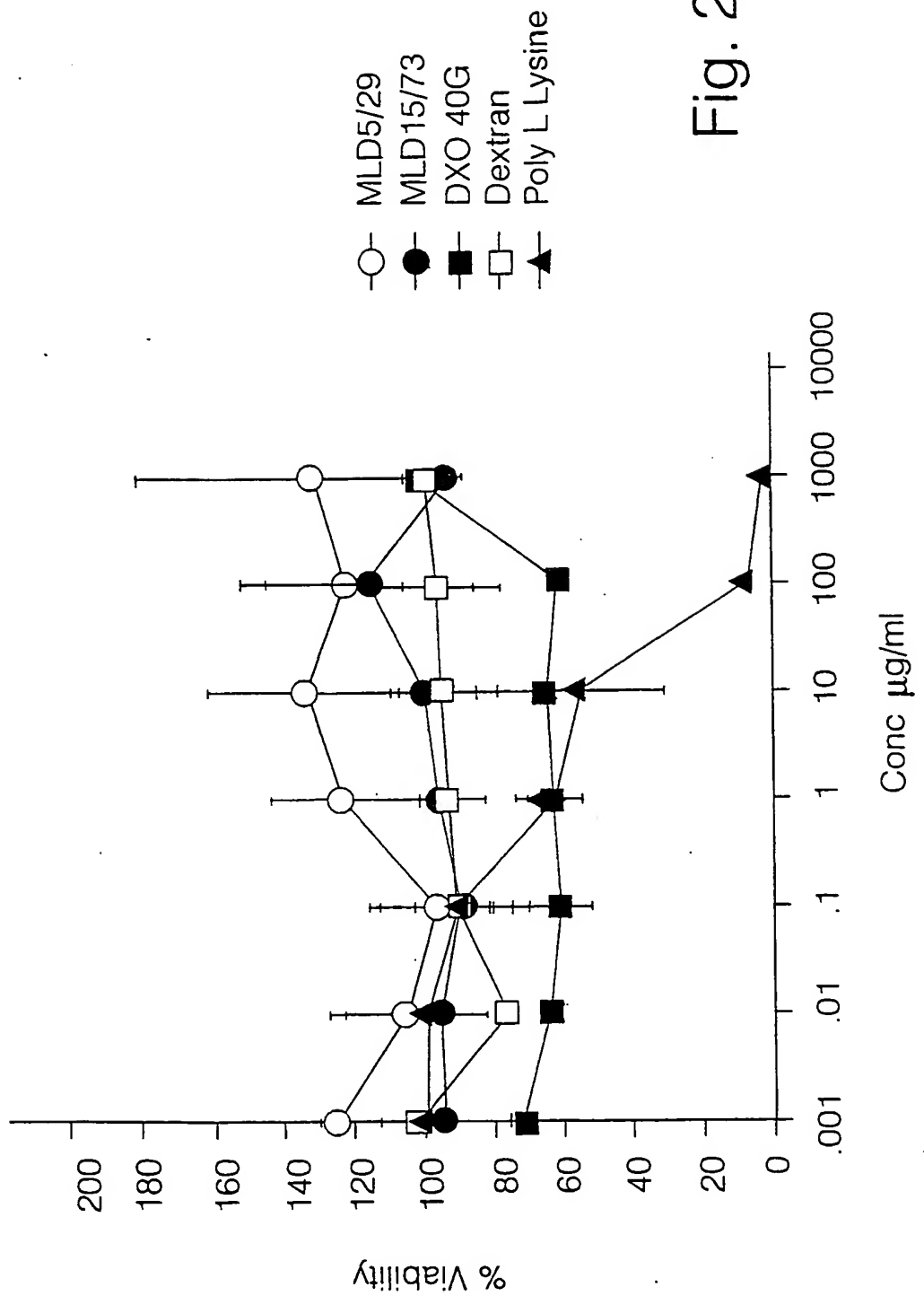


Fig. 2

3/15

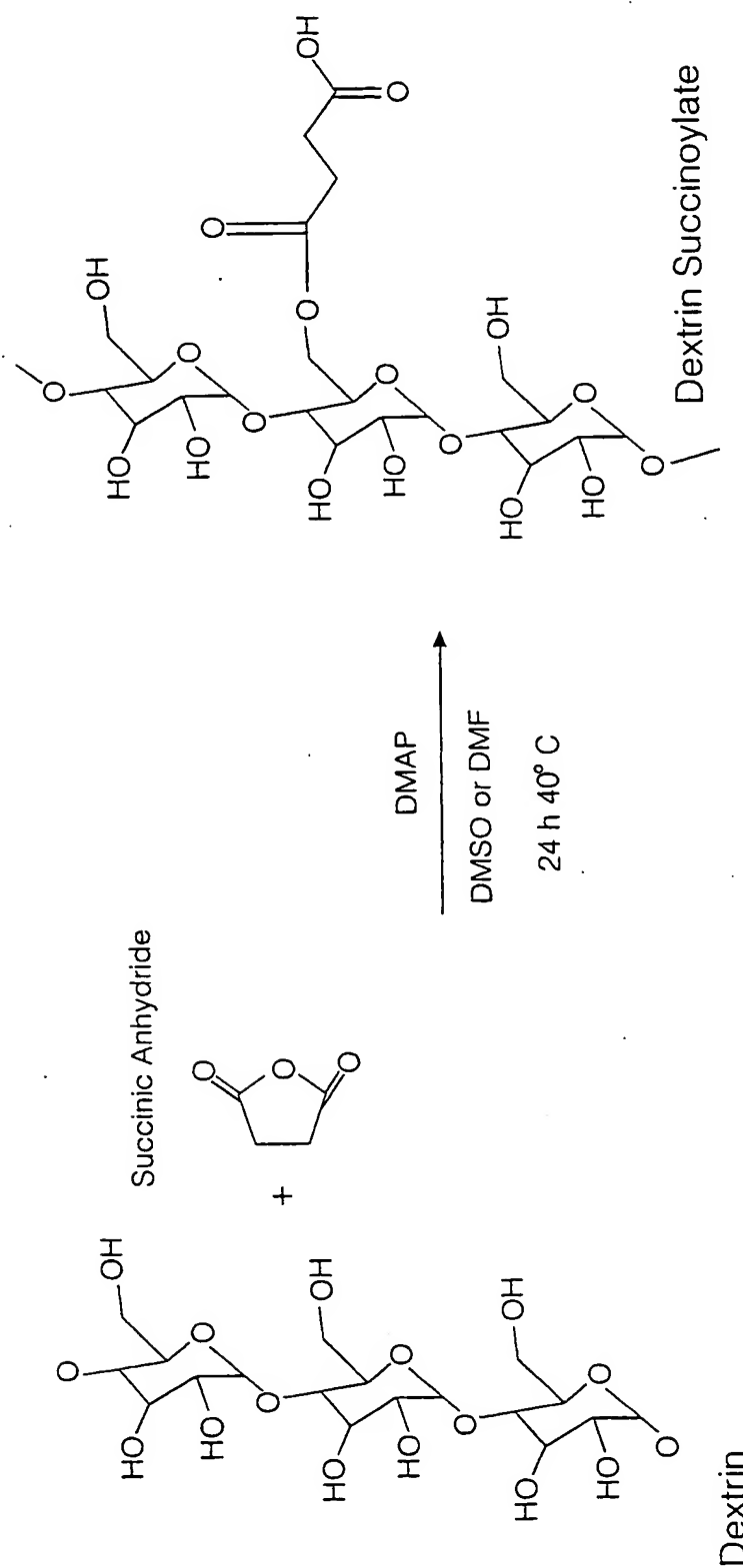


Fig. 3A

4/15

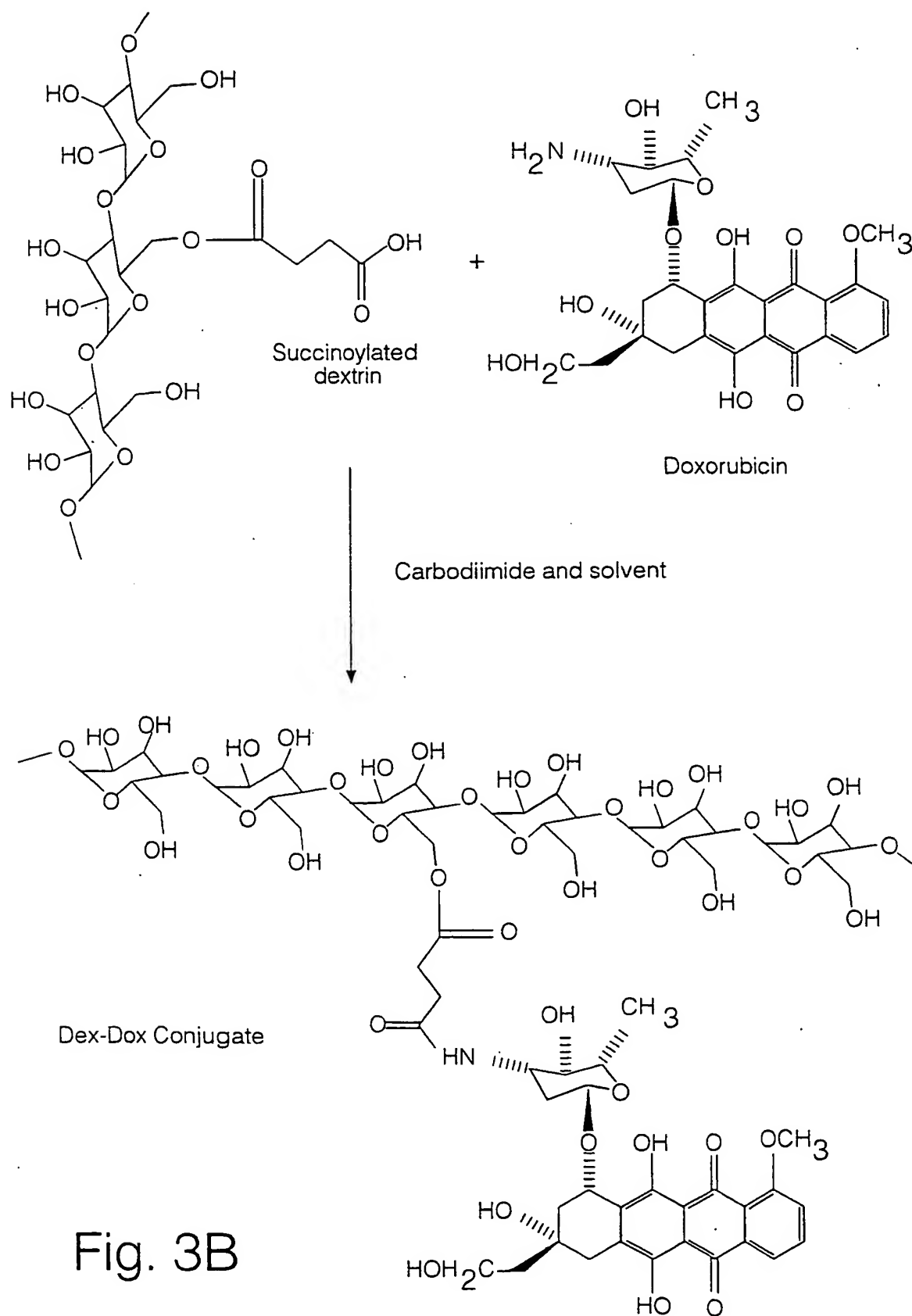
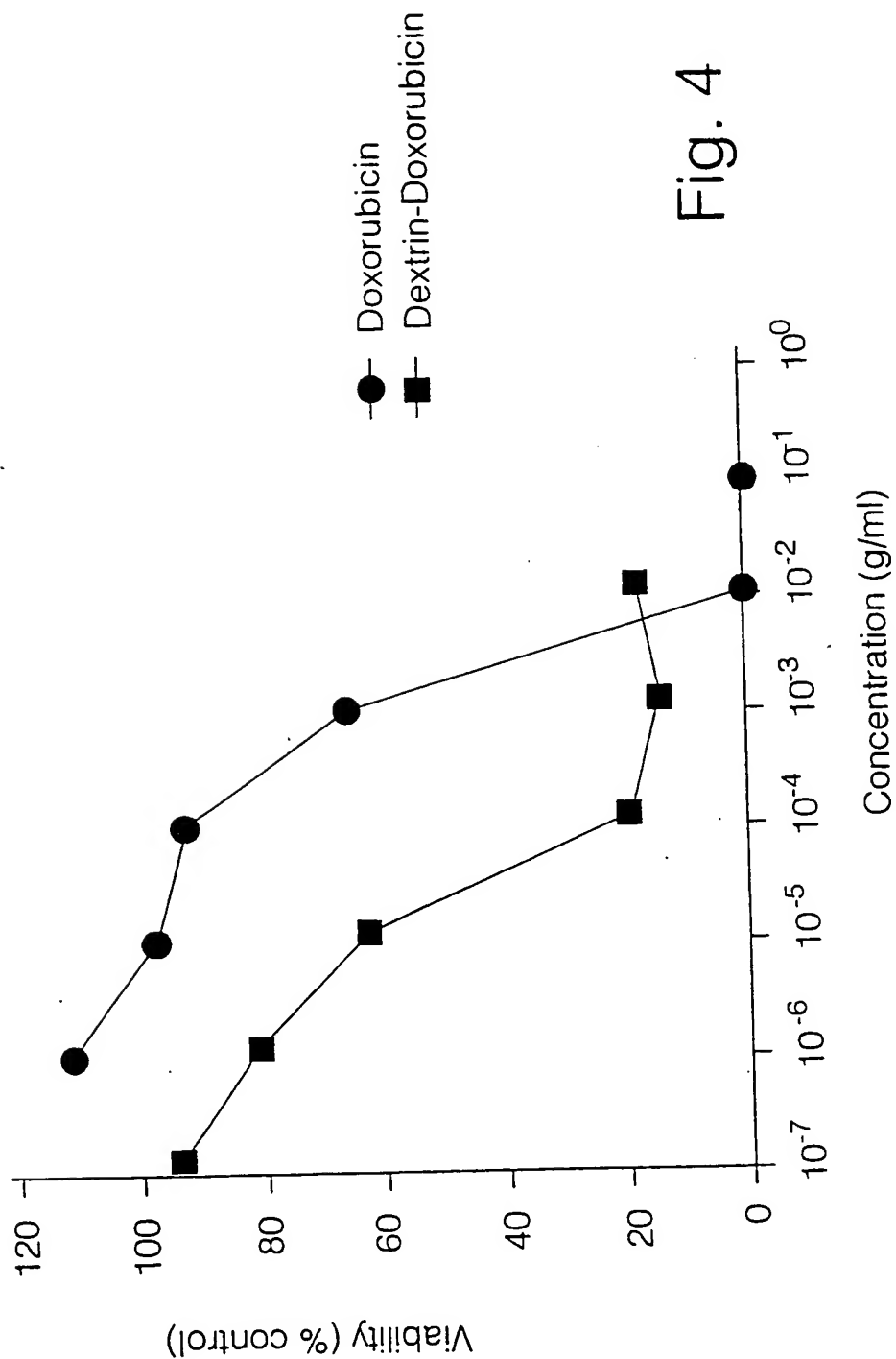


Fig. 3B

5/15



SUBSTITUTE SHEET (RULE 26)

6/15

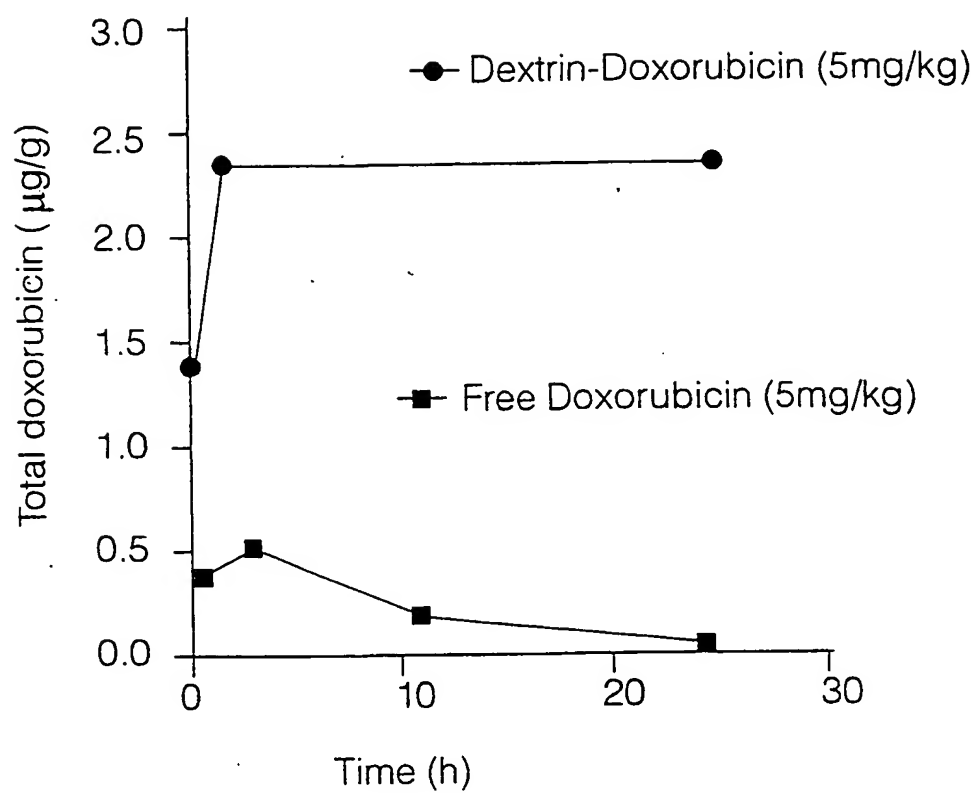


Fig. 5

7/15

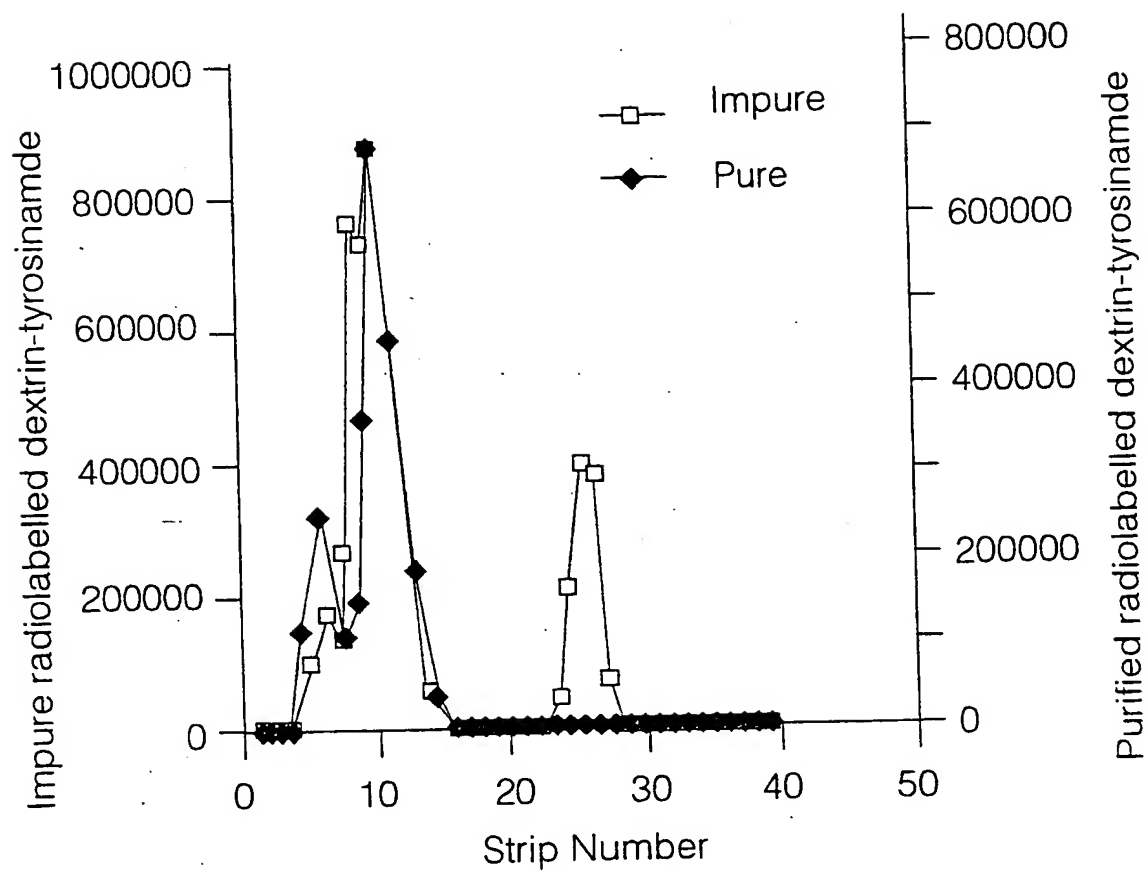


Fig. 6

8/15

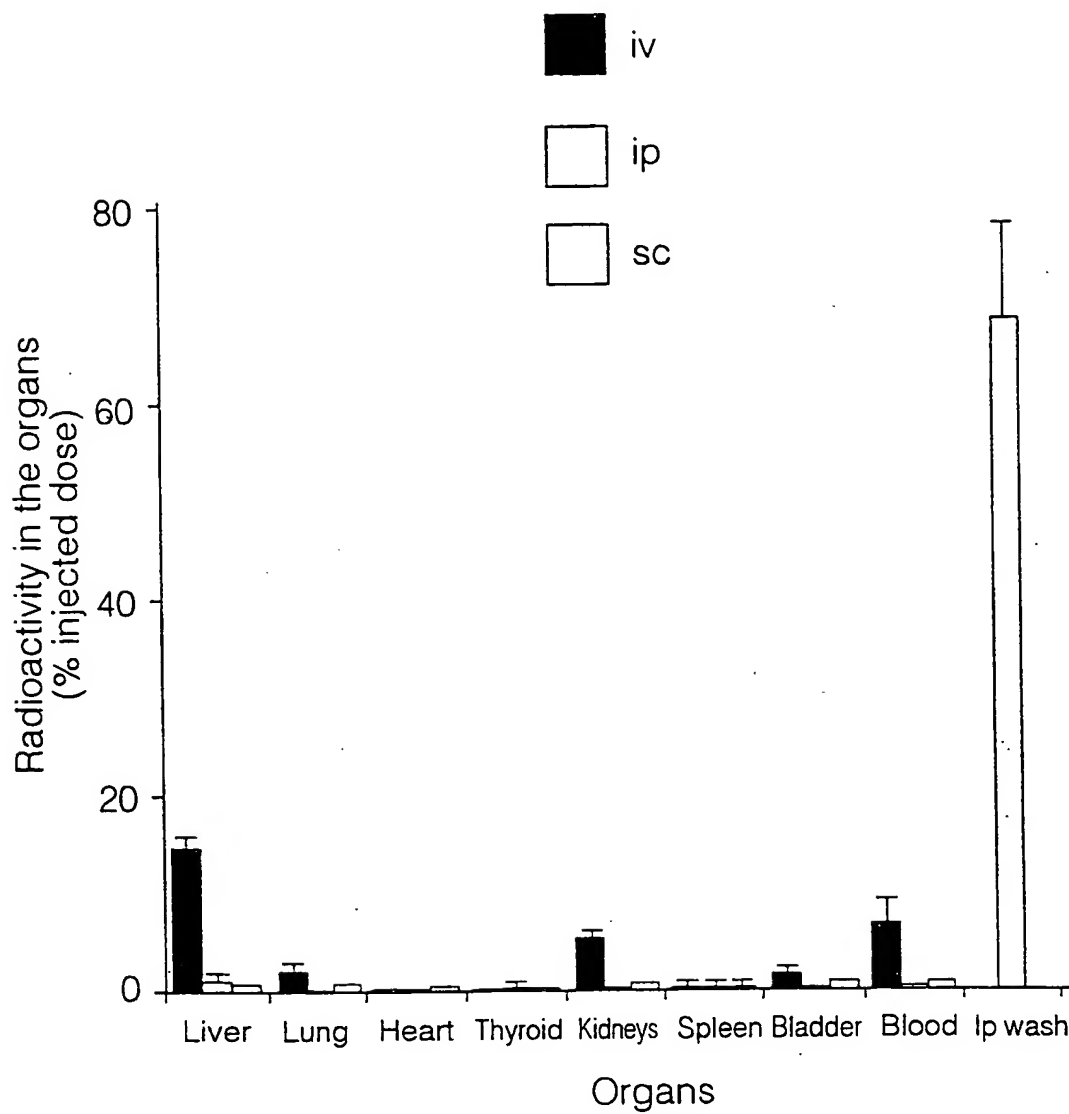


Fig. 7A

9/15

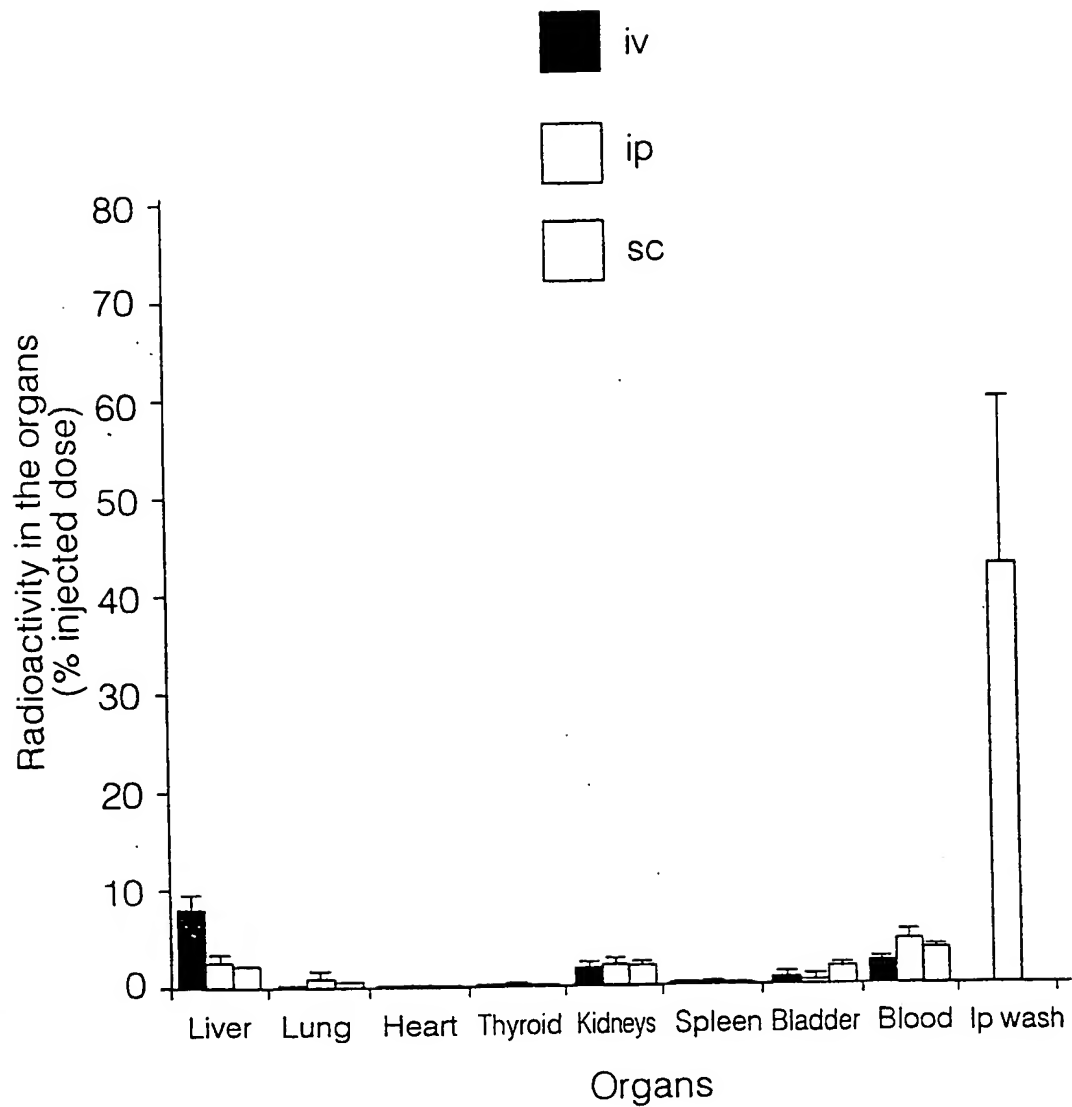


Fig. 7B

10/15

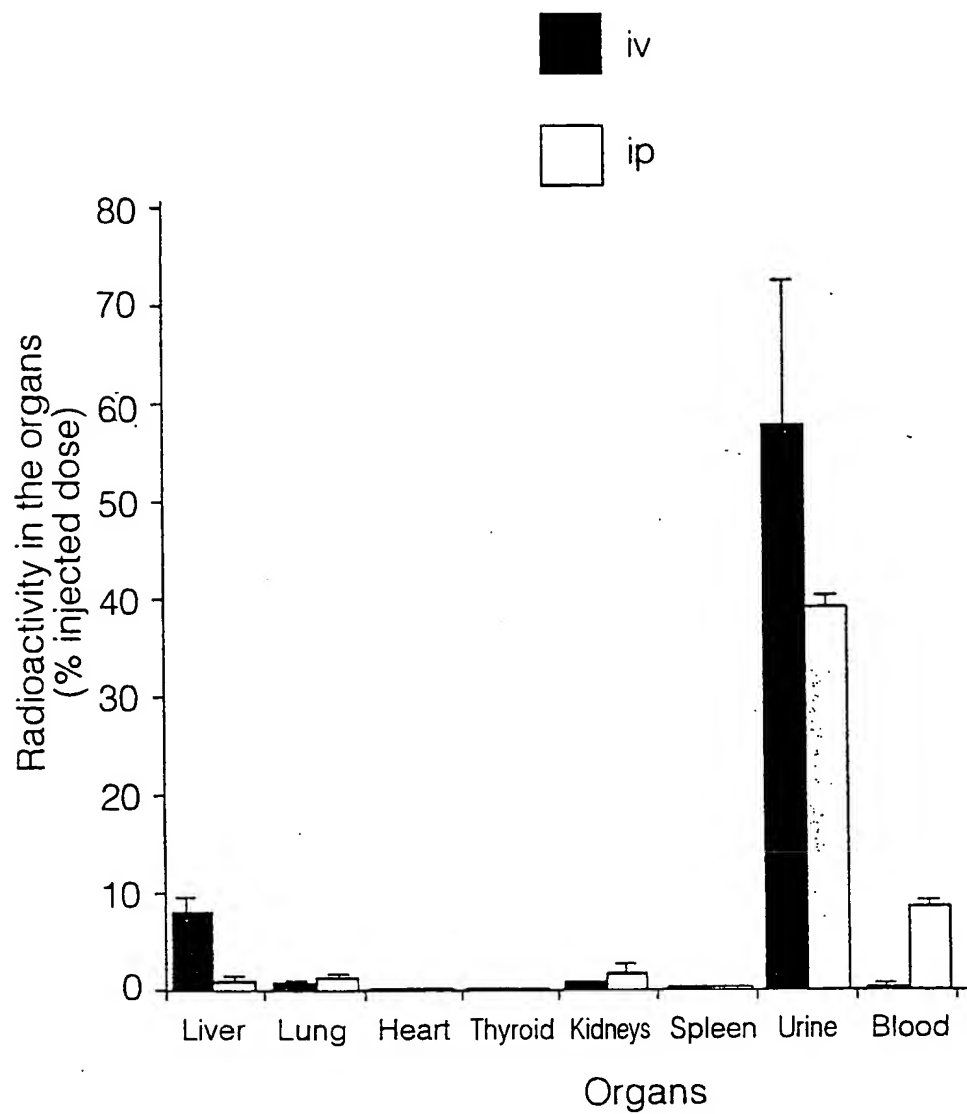


Fig. 7C

11/15

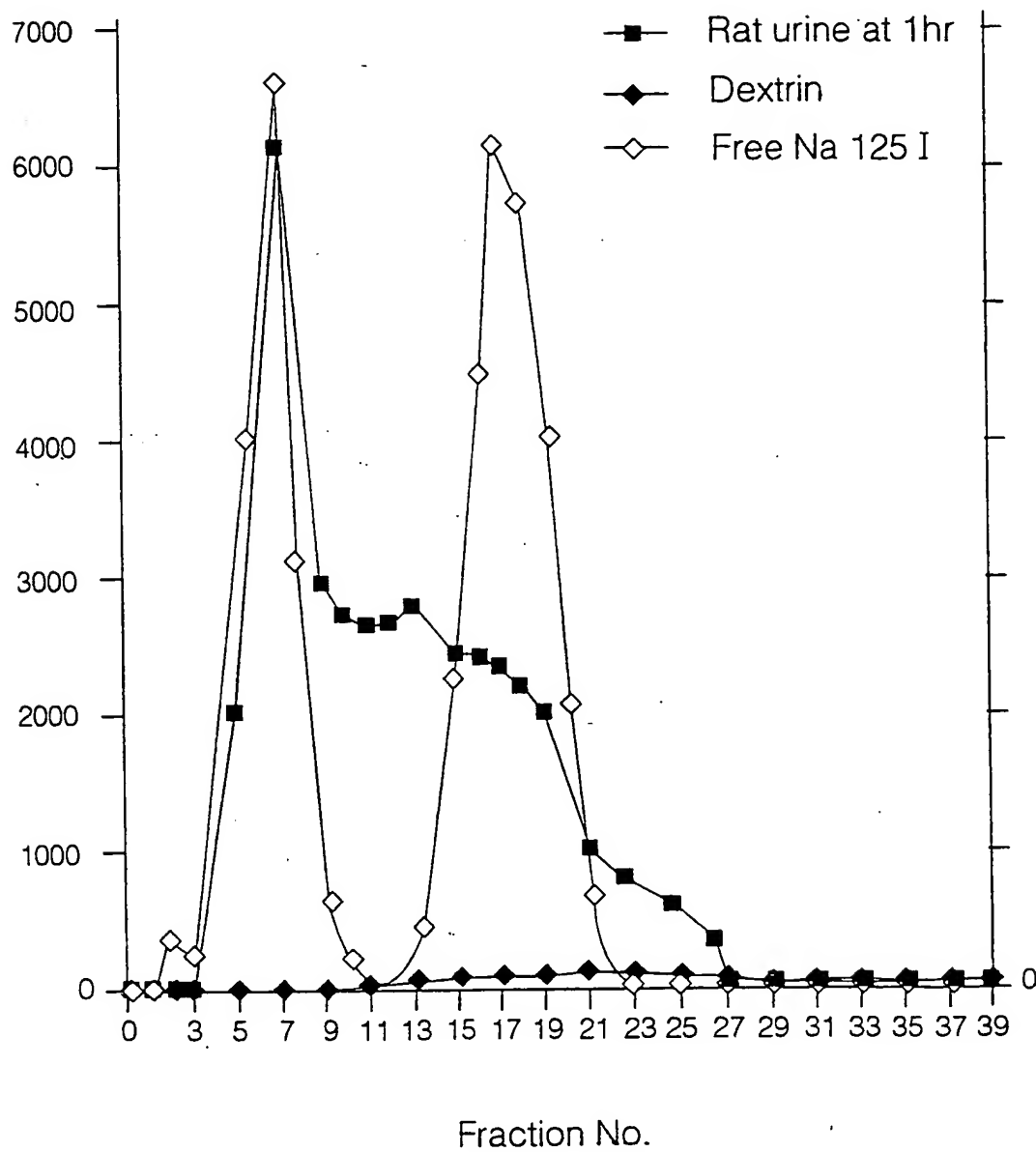


Fig. 8

12/15

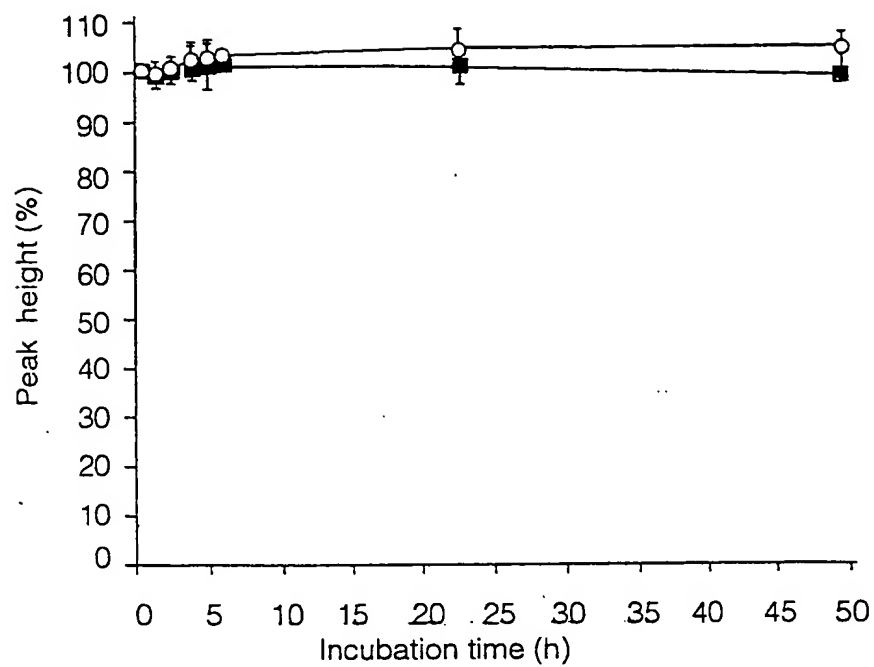


Fig. 9

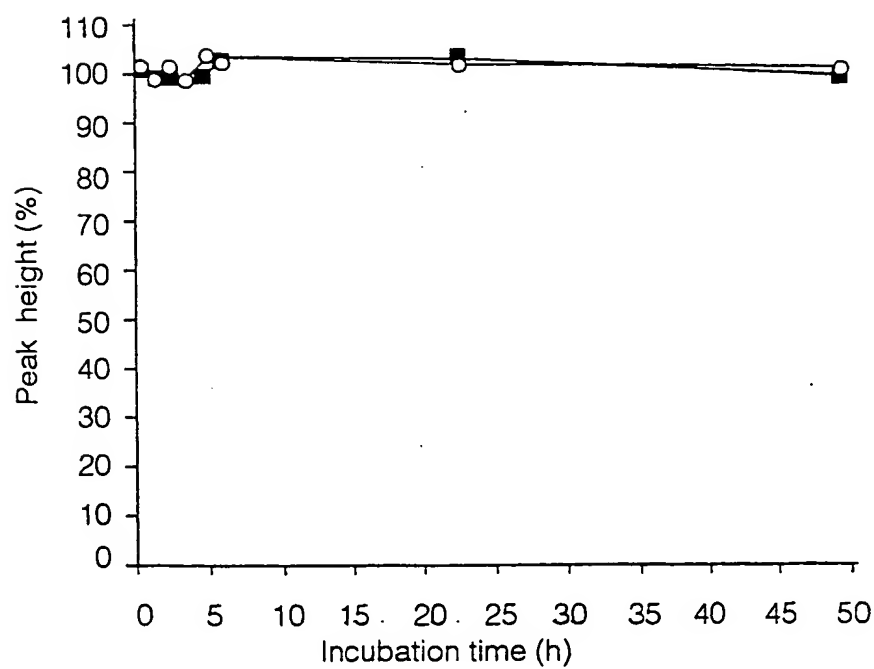


Fig. 10

13/15

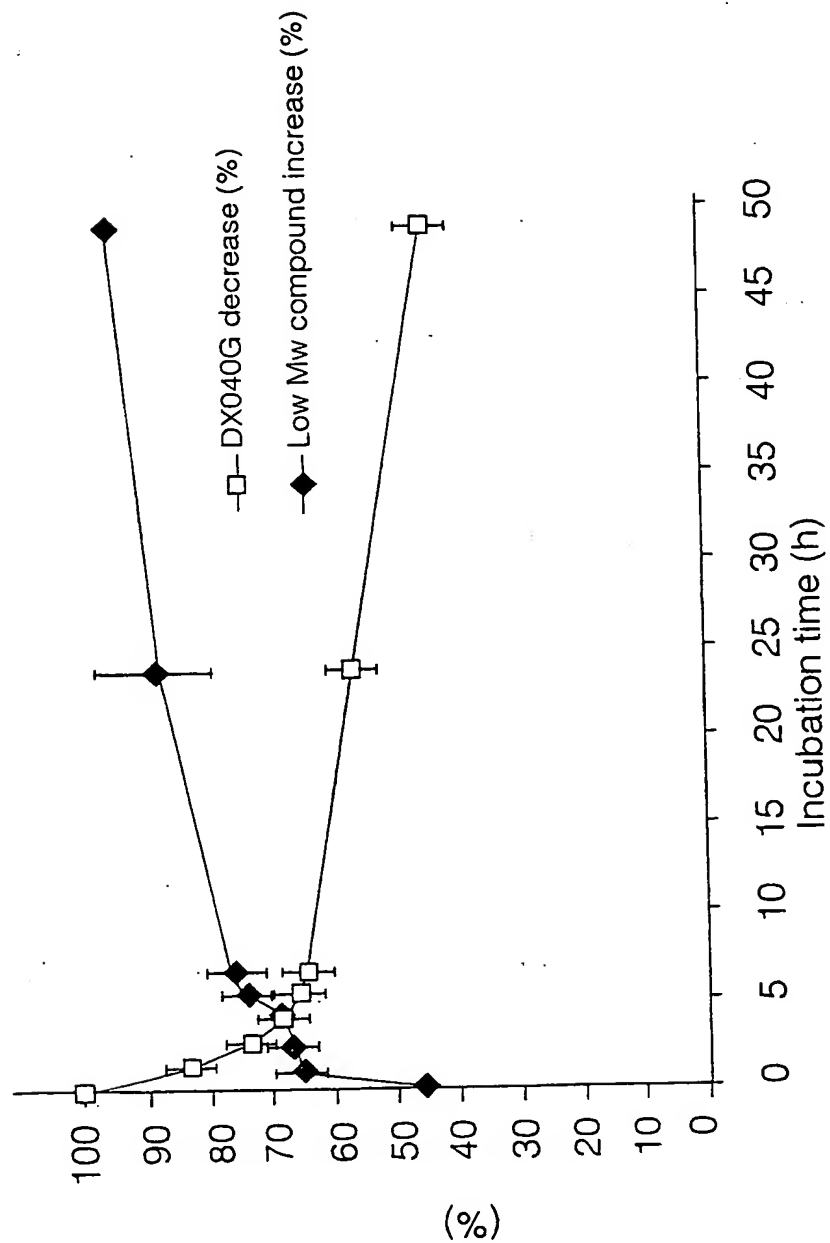


Fig. 11

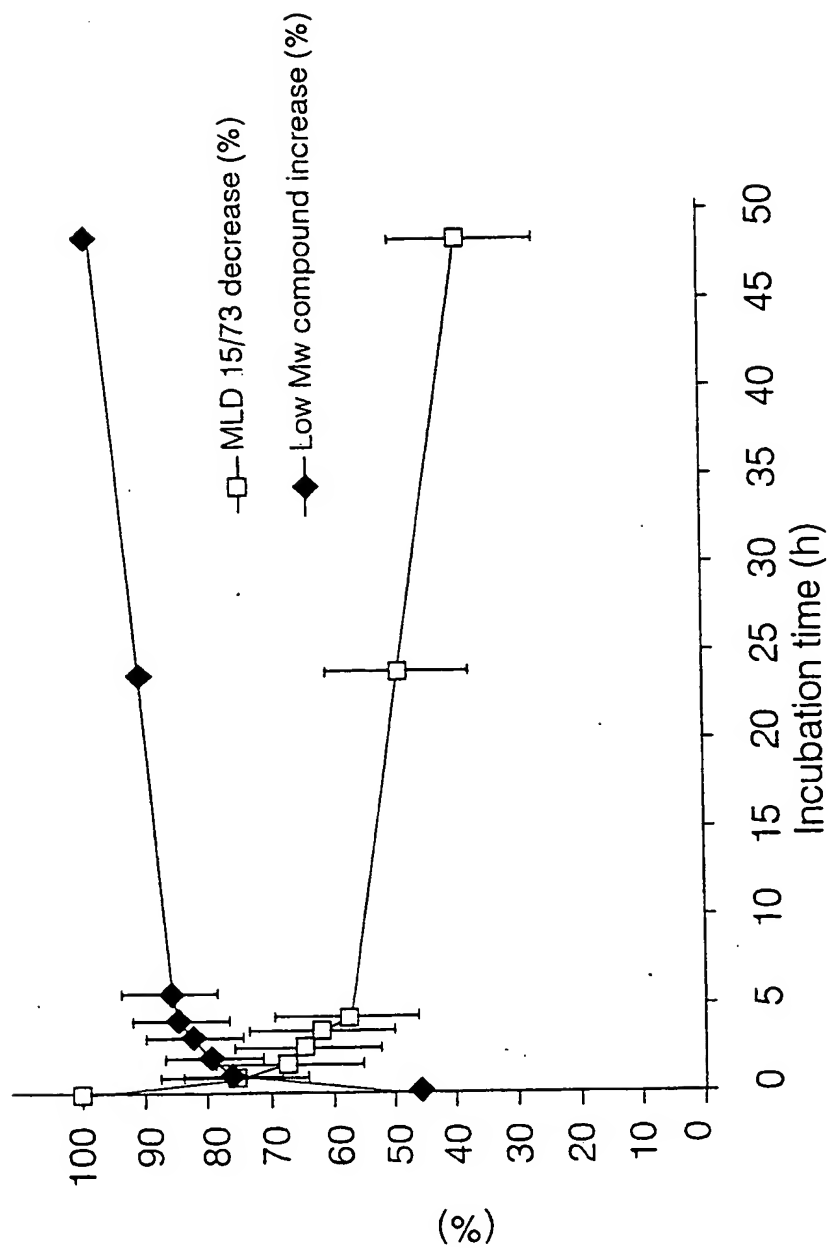


Fig. 12

15/15

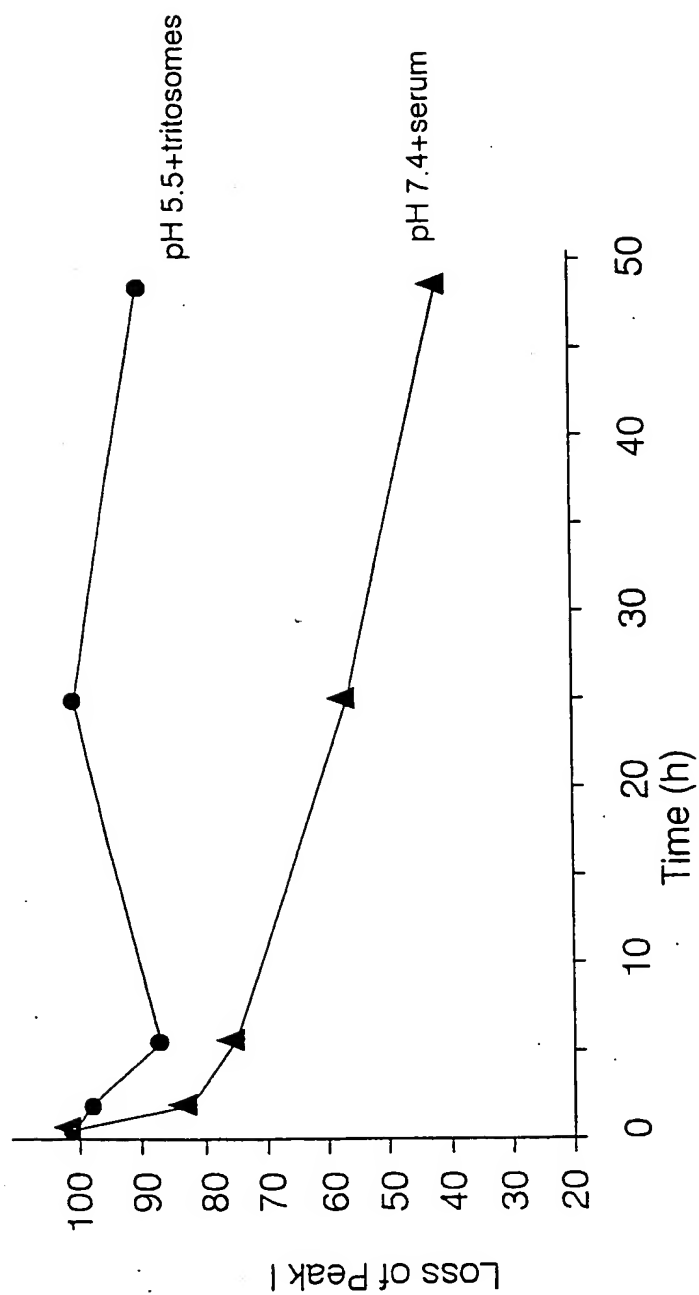


Fig. 13

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/GB 98/01567

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K47/48

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	R. DUNCAN ET AL.: "Dextrins as Carriers of Anticancer Agents" PROC. INT. SYMP. CONTROLLED RELEASE BIOACT. MATER., 24TH, 15 June 1997, pages 771-772, XP002078115 see the whole document ---	1-14, 18-30
X	DATABASE CHEMABS CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US. AN: 120:135152 KAWASAKI, AKIO ET AL: "Preparation of protein-polysaccharide conjugates as pharmaceuticals and cosmetics" XP002078120 see abstract & JP5339298 A (Taiyo Kagaku Kk, Japan) 21-12-1993 --- -/--	1, 3

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

21 September 1998

Date of mailing of the international search report

08/10/1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.
Fax: (+31-70) 340-3016

Authorized officer

Veronese, A

INTERNATIONAL SEARCH REPORT

Internatio Application No
PCT/GB 98/01567

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO 95 05199 A (DEXTRAN PRODUCTS LTD) 23 February 1995</p> <p>see page 3, line 2-9 see page 5, line 10 - page 7, line 16 * See figures pag. 8-10 * see claims 1,7</p> <p style="text-align: center;">---</p>	<p>1,3-7, 10,13, 14,18-23</p>
X	<p>US 4 496 689 A (MITRA GAUTAM) 29 January 1985</p> <p>see claims 1,3,6</p> <p style="text-align: center;">---</p>	<p>1,3-7, 10,13, 14,18-23</p>
X	<p>WO 95 34325 A (ADVANCED MAGNETICS INC) 21 December 1995</p> <p>*See abstract and Figure 4 * see claims 1,30,33</p> <p style="text-align: center;">---</p>	<p>1,3-7, 10,13, 14,18-23</p>
X	<p>EP 0 540 751 A (EISAI CO LTD) 12 May 1993</p> <p>see page 2, line 39; claim 3</p> <p style="text-align: center;">---</p>	<p>1,3-7, 10,13, 14,18-23</p>
Y	<p>FR 2 346 016 A (FERRUTI PAOLO) 28 October 1977</p> <p>see page 2, line 19-24; claims 1-6; example 3</p> <p style="text-align: center;">---</p>	<p>1,3-5, 10,12</p>
Y	<p>P.H.LIST AND L. HORHAMMER: "HAGERS HANDBUCH DER PHARMAZEUTISCHEN PRAXIS" 1972, SPRINGLER VERLAG, BERLIN-HEILDELBERG-NEW YORK XP002078119 * See volume 3, pag. 61-65: "Amylum solubile" and "Dextrinum" *</p> <p style="text-align: center;">---</p>	<p>1,3-5, 10,12</p>
A	<p>"Thermostability of soluble and immobilized subtilisins after their modification by dextrans and dextrans." ENZYME ENG., no. 5, - 1980 pages 423-426, XP002078116 see page 423 - page 424; figure 1</p> <p style="text-align: center;">---</p>	
A	<p>GERRIT H P. ET AL.: "Preparation, Characterization and Pharmaceutical Application of Linear Dextrans. II. Complexation and Dispersion of Drugs with amyloextrin by Freeze-Drying and Kneading." PHARMACEUTICAL RESEARCH, vol. 10, no. 9, 1993, pages 1280-1284, XP002078118 see the whole document</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	<p>1-30</p>

INTERNATIONAL SEARCH REPORT

Internatio Application No

PCT/GB 98/01567

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	L.W.SEIMOUR E AL.: "Tumour tropism and anti-cancer efficacy of polymer-based doxorubicin prodrugs in the treatment of subcutaneous murine B16F10 melanoma" BR.J.CANCER, vol. 70, 1994, pages 636-641, XP002078117 see the whole document -----	1-30
A	WO 92 04904 A (ML LAB PLC) 2 April 1992 see the whole document -----	16,17

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 98/01567

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9505199 A	23-02-1995	NONE	
US 4496689 A	29-01-1985	CA 1242664 A EP 0147761 A JP 1970305 C JP 6096541 B JP 60178823 A	04-10-1988 10-07-1985 18-09-1995 30-11-1994 12-09-1985
WO 9534325 A	21-12-1995	US 5554386 A AU 2700195 A CN 1150761 A US 5589591 A ZA 9504925 A	10-09-1996 05-01-1996 28-05-1997 31-12-1996 13-02-1996
EP 0540751 A	12-05-1993	WO 9220699 A	26-11-1992
FR 2346016 A	28-10-1977	DE 2546242 A GB 1527351 A GB 1526720 A GB 1526795 A JP 945056 C JP 52114688 A JP 53024471 B SE 431550 B SE 7603070 A US 4067876 A US 4169923 A US 4166902 A	29-04-1976 04-10-1978 27-09-1978 27-09-1978 20-03-1979 26-09-1977 20-07-1978 13-02-1984 09-09-1977 10-01-1978 02-10-1979 04-09-1979
WO 9204904 A	02-04-1992	AT 165005 T AU 649731 B AU 8614591 A DE 69129272 D EP 0550532 A ES 2113888 T JP 6503305 T PT 99057 A US 5439892 A	15-05-1998 02-06-1994 15-04-1992 20-05-1998 14-07-1993 16-05-1998 14-04-1994 31-08-1992 08-08-1995

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☒ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.